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Review article

Strategy for the production of human monoclonal antibodies using in vitro activated B cells

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An up-to-date strategy to optimally produce human monoclonal antibodies by primary in vitro immunization or by secondary in vivo stimulation of immunized donors is discussed in detail. The effect of a lysosomotropic amino acid dipeptide on the B cell suppression by lysosome-rich cytolytic cells and the subsequent Epstein-Barr virus transformation of immune B lymphocytes is explained. The described strategy allows a routine production of human hybridomas, derived from peripheral blood lymphocytes and exhibiting a productivity in the range of 20–50 μg Ig/24 h per 10^6 cells. Furthermore, the possibilities to modulate antibody isotype and affinity by molecular biological methods is reviewed.

Key words: Hybridoma, human; Monoclonal antibody; Leucyl-leucine methyl ester; In vitro immunization; Epstein-Barr virus transformation; Polymerase chain reaction

Introduction

High expectations on human monoclonal antibodies as the solution to problems connected with antibody mediated immunotherapy have been expressed by several investigators (for review, see Larrick and Bourla, 1986; James and Bell, 1987). To this date, however, only very limited experience using human monoclonal antibodies in in vivo trials has been reported (Philips et al., 1982; Irie and Morton, 1986; Burnett et al., 1987) and the full clinical potential of these antibodies remains to be further investigated. The suggested, and partially verified, advantages of human monoclonal antibodies, as compared to mouse antibodies, are: first, they drastically reduce an anti-Ig response when used in vivo in patients. Sensitization of patients with foreign mouse proteins have in several investigations been shown to give a number of side effects such as fever, rashes, vomiting, urticaria, bronchospasm, tachycardia, dyspnea, etc. These side effects are normally transient and dissappear as soon as the infusion of mouse antibodies is interrupted. However, much more serious is the fact that the efficiency of the immunotherapy is severely reduced since mouse monoclonal antibodies are complexed by the humoral anti-immunoglobulin response mounted by the patient receiving the treatment. Secondly, the asparagine-linked carbohydrate sequences of human monoclonal antibodies are more compatible with Fc receptors on human effector cells, as compared with carbohydrate sequences on mouse antibodies. Thirdly, a different repertoir of antibody specificities can be obtained in human monoclonal antibodies; xenogeneic immunizations predominantly give antibodies against immunodominant membrane-bound antigenic structures, like blood group substances, monomorphic

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(frame work) epitopes on human histocompatibility antigens, etc. In contrast, human antibodies tend to recognize polymorphic epitopes on histocompatibility antigens (Effros et al., 1986), tumor-associated antigens – 'altered self' antigens – and other determinants that the murine immune system neglects (Schlom et al., 1980; Ohlin et al., 1989a). Despite the fact that tumor-specific antigens have not been identified, except for the idiotypic T and B cell receptors in lymphoproliferative diseases, this implies that the human immune response to autochthonous tumors could be unique and that even small alterations in antigen structures will be discovered. This would enable us to obtain completely new and more fine-tuned specificities in human monoclonal antibodies as compared to mouse monoclonal antibodies. However, this fact might limit a more general use of the technology to genetically engineer antibodies (Jones et al., 1986) by combining mouse gene segments coding for the complementarity determining regions with segments coding for constant regions of a human antibody, since the specificity of these chimeric antibodies will still be determined by the mouse immune system.

Below, I will briefly describe the present problems in human hybridoma technology and in detail discuss the present strategy to solve the most important and how to optimally obtain antigen-specific antibodies. For more comprehensive covarage of the overall area of human hybridomas, I refer to the following reviews (Engleman et al., 1985; James and Bell, 1987; Borrebaeck, 1988c).

Present problems in human hybridoma technology

Almost a decade has passed since the first reports on human monoclonal antibodies were published and some of the past production problems have been solved although several still remain the same (Table I). Significant research efforts have been focussed in problems 1 and 2 and based on previous experience with mouse splenocytes (reviewed by Borrebaeck, 1986, 1988a) recent progress using human B lymphocytes seems to pave the way to routinely perform in vitro immunizations for the production of human antibodies (reviewed in Borrebaeck, 1988b). This is

TABLE I

PAST AND PRESENT PROBLEMS IN HUMAN HYBRIDOMA TECHNOLOGY

1. Source of human B lymphocytes
2. In vitro immunization of human B lymphocytes
3. Immortalization of immune human B lymphocytes
4. Stability of human hybridoma cell lines
5. Modulation of isotype and affinity of the human antibody
6. Production and purification of multi-gram quantities of monoclonal antibody

absolutely necessary since totally very few antigens can be utilized in a situation where human individuals deliberately are being immunized. In vitro immunization is the induction of an antigen-specific, primary immune response and results in the production of IgM antibodies, exhibiting binding constants in the range 10^6–10^8 M^{-1}. However, in vitro immunization technology can also preferably be used to effectively induce a secondary, antigen-driven immune response in preactivated B cells and results in the production of primarily IgG antibodies, exhibiting binding constants $> 10^9$ M^{-1}.

Different approaches to immortalization are described in detail by Jonak and co-workes (1988) and Kozbor (1988), using DNA transfection or Epstein-Barr virus infection of B cells, respectively. The stability problem of human hybridoma cell lines has, through the years, been very pronounced and the majority of human hybridomas found in the literature do not exist today. This problem might be overcome either by the new and more stable fusion partners that have emerged in recent years or preferably by different ways of immortalizing.

Modulation of isotype and affinity of the produced human monoclonal antibody is one problem that by preference can be solved by genetic engineering of a preformed human monoclonal antibody. Using this technology, the gene segment coding for the constant region of the heavy chain can be changed and an isotype switch from IgM to IgG can be achieved, thereby obtaining a chimeric human/human antibody (Morrison, 1985). Furthermore, an increased antibody affinity might be obtained by applying site-directed mutagenesis to the heavy and light chain variable re-

gions of monoclonal antibodies (Roberts et al., 1987).

Finally, production of purification of multigram quantities of human monoclonal antibodies are problems where the experience still is limited and routine solutions to these problems await further investigations and access to a variety of human hybridomas. For economic feasibility as well as for in vivo therapeutic antibody applications, the human monoclonal antibodies have to be produced by adaptation of the hybridomas to growth in animal cell fermentors or large-scale-culture equipment based on hollow fiber/ceramic core units.

How to avoid problems in human hybridoma technology

Source of human B lymphocytes

The most readily available lymphoid compartment is by far peripheral blood. Compared to other sources obtained by surgery, e.g., spleens, tonsils, or lymph nodes, peripheral blood easily lends itself to repeated sampling from the same individual. However, peripheral blood has consistently performed very poorly when used for the production of both mouse and human monoclonal antibodies and has been considered to be a suboptimal source of lymphoid cells (Olsson et al., 1983; Lagacé and Brodeur, 1985; Ho, 1987; James and Bell, 1987). The reason for this might be the failure to realize that human B cells found in the peripheral blood could be under more severe suppression compared to B cells in, e.g., the spleen and lymph nodes, since the role of the former lymphoid compartment is not to act as an activation site but rather as a transport system connecting both central and peripheral lymphoid organs. Furthermore, one need to investigate the activation pathways of B cells of different lymphoid origins, since, e.g., the lymphokine requirements may vary depending on what lymphoid compartment the cells are isolated from (Jelinek and Lipsky, 1987). To finally become a generally available technology, it must, however, be possible to routinely produce human monoclonal antibodies using peripheral blood lymphocytes (PBL), since the availability of surgically removed lymphoid organs is very scarce in large parts of the Western world. To investigate the applicability of human PBL for the production of human monoclonal antibodies, we recently determined the lymphokine requirements during in vitro activations of PBLs (Danielsson et al., 1987). The results clearly showed that peripheral B lymphocytes were not antigen specifically activated if cultured in the presence of only antigen and various lymphokines. It was necessary to separate individual cell populations and reconstruct a mixture of T, B and accessory cells at a fixed ratio before an antigen-specific activation could be recorded (Danielsson et al., 1987). This supports the fact that peripheral B lymphocytes are subjected to a cell-mediated supressive restraint more severe than, e.g., splenic B cells.

In vitro immunization of human B lymphocytes

The clonotype frequency of epitope-specific B cells in human peripheral blood has been estimated by limiting dilution experiments to 10^{-4}–10^{-5}, depending on the immune status of the donor (Yarchoan et al., 1981; Carson and Freimark, 1986; Yamaura et al., 1986). Theoretically, if an in vitro immunization culture of human peripheral blood lymphocytes consists of totally 10^8 cells, approximately 10^3 of these are consequently clonotype specific. However, due to an antigen-specific clonal expansion during an in vitro immunization period, each B cell might expand to approximately $1–3 \times 10^2$ cells (using a culture period of 6–7 days and a B cell generation time of 20 h). Starting with 10^8 cells, this would (on day 7) at best give us approximately 3×10^5 cells of a specific clonotype. This points to the need of alternative immortalization techniques that allow us to rescue a small amount of specific B cells, since the fusion frequency of human-human or human-mouse hybridoma system is 1 in 10^5–10^6 cells (Abrams et al., 1983).

Danielsson and co-workers (1987) recently investigated the requirements of cytokines during an in vitro immunization and clonal expansion of human peripheral blood lymphocytes. To be able to antigen-specifically activate any of the peripheral B cells at all, the blood mononuclear cell population had to be separated into subpopulations consisting of T, B or accessory cells (A cells);

TABLE II

THE EFFECT OF DIFFEENT CYTOKINES ON THE NUMBER OF PLAQUE-FORMING CELLS IN A 5 DAY ANTIGEN-SPECIFIC IN VITRO CULTURE OF HUMAN SEPARATED PBL (Danielsson et al., 1987), USING 2 μg KLH/ml AS ANTIGEN

	KLH	sPWM-T [a]	IL-2 [b]	no. of plaque forming cells/10^6 B cells [c]		
				KLH	Ovalbumin	Gelatin
(1)	+	−	−	0	0	0
(2)	+	+	−	105	15	1
(3)	+	+	+	170	23	0
(4)	−	+	+	7	8	1
(5)	+	−	+	0	0	0

[a] sPWM-T: 25% supernatant from PWM stimulated (10 μg PWM/ml for 24 h), irradiated (2000 rad) human T cells.
[b] IL-2: 5 U/ml.
[c] Möller and Borrebaeck, 1985.

unseparated cells could not be antigen-specifically activated. A recombination of the purified cell populations at the ratio 0.25 : 1 : 2 of A, B and T cells, respectively, was then shown to give 100–200 specific plaque-forming cells/10^6 B cells. The effect of recombinant IL-1, IL-2, and IFN-γ together with BCDF and sPWM-T were tested using this combination of T, B, and A cells (Table II). The presence of sPWM-T was shown to be crucial for an antigen-specific activation of peripheral B cells. The addition of exogenous IL-2 further increased the number of plaque-forming cells, whereas IL-1 and BCDF had no effect in the described system (Danielsson et al., 1987). These results pointed to the important fact that human B lymphocytes were subjected to suppression from other cell populations present in peripheral blood, which prevented the B cells from being antigen-specifically activated unless the suppressive subpopulations were removed.

A number of different cell types, e.g., $CD8^+$ or $CD8^+/CD11^+$ T cells, large granular lymphocytes, cytotoxic T cells, monocytes, etc., were investigated in an attempt to identify what cell population(s) that was involved in the suppression of an antigen-specific activation of peripheral B lymphocytes. Finally, a lysosomotropic methyl es-

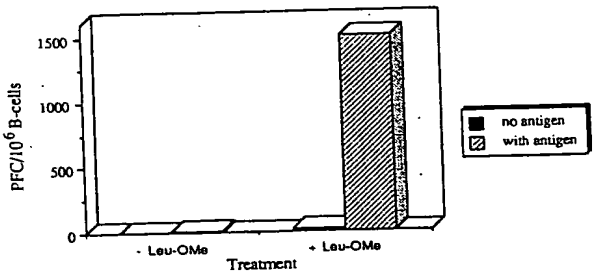

Fig. 1. Effect of treating human peripheral lymphocytes with leucine methyl ester (Leu-OMe) before an in vitro immunization, using 1 μg KLH/ml. The culture was also supplemented with 5 U IL-2/ml and sPWM-T (25%), cultured for 6 days and tested using a plaque assay (Möller and Borrebaeck, 1985). The plaque number is the mean of triplicate assays.

TABLE III

THE EFFECT OF LEUCYL-LEUCINE METHYL ESTER (LL) ON HUMAN PERIPHERAL LYMPHOCYTES, MEASURED 18 h AFTER TREATMENT

The values are given as % of total number of cells counted. Leu7 reacts predominantly with natural killer cells and a subset of T cells; CD16 defines the Fc receptor on natural killer cells and neutrophilic granulocytes; CD14 defines monocytes; CD20 defines B cells; CD45R defines a 220 kDa antigen found on NK cells, B cells, and T cells; CD4/CD45R defines suppressor/inducer T helper cells. Adapted from Ohlin et al. (1989b).

Experiment:	no. 1		no. 2	
treatment:	−	LL	−	LL
Marker				
Leu7	2.1	0.3	4.9	0.2
CD16	5.3	0.2	7.5	0.3
CD14	19	0.2	11	0.2
CD8	32	29	34	24
CD4	61	73	47	64
CD20	8.3	6.5	17	14
CD4/CD45R	49	54	29	40
CD8/CD11	6.0	0.0	6.0	0.0

ter of leucine or its dipeptide leucyl-leucine, initially described by Thiele and Lipsky (1985a,b), was shown to have a profound effect on cells that were down-regulating the antigen-specific activation of peripheral blood B lymphocytes (Fig. 1 and Table III (Borrebaeck et al., 1987; Ohlin et al., 1989b). The suppression was thus mediated by lysosome-rich natural killer (suppressor?) cells, monocytes, and a subset of cytotoxic $CD8^+$ T cells.

In summary, the necessary treatment of PBL is performed using 125–250 μM of leucyl-leucine methyl ester in serum-free medium for 15 min (Ohlin et al., 1989b). The cell yield is normally 65% ($n = 35$), although it might vary between 40–90% depending on the individual buffy coat. If much lower yields are consistently achieved the batch of dipeptide should be changed, since this chemical is aging which may result in an excessive cell death. The treated peripheral blood lymphocytes are then cultured at a cell density of 3.5×10^6 cells/ml of supplemented RPMI 1640, containing 10% human ABO serum, 25% sPWM-T, and 5 U IL-2/ml. The culture period is 6 days and the antigen concentration should be in the range 10–500 ng/ml; it is very important to test several antigen concentrations, e.g., 25, 125, and 500 ng/ml, when starting with a new immunogen since the optimal amount differ between antigens.

The described primary in vitro immunization technology using leucine methyl ester-treated peripheral blood lymphocytes has allowed the production of human monoclonal antibodies against a number of different antigens, e.g., recombinant peptides from gp120/41 of HIV (Borrebaeck et al., 1988d; Ohlin et al., 1989a), digoxin (Borrebaeck et al., 1987), the Thomsen-Friedenreich antigen (Jansson and Borrebaeck, unpublished data), trombocytic antigens (Hagen et al., unpublished data), *Mycoplasma hominis* (Möller and Borrebaeck, 1989), etc. Furthermore, the same immunization system can preferentially be used to induce a secondary in vitro stimulation thus clonally expanding in vivo preactivated B cells against, e.g., viral antigens. The technology, as described above, has been applied to in vitro stimulation of cells derived from seropositive individuals and the production of numerous hybridomas secreting specific antibodies against cytomegalovirus has been recorded (Ohlin and Borrebaeck, unpublished data).

Immortalization of immune human B lymphocytes

Presently, the most frequent way to immortalize immunized human B cells is by conventional PEG fusion or by an initial infection using the B lymphotropic herpesvirus, Epstein-Barr virus, followed by a subsequent PEG fusion, a technique introduced by Kozbor and co-workers (1982). As discussed previously, the immortalization frequencies are crucial to improve the probability to obtain a high yield of desired antigen-specific hybridomas or lymphoblastoid cell lines. Compared to PEG-fusion, the EBV infection has been reported to give several orders of magnitude more favorable fusion frequency and an infection rate of over 1% of the B cells is not unusual (Engleman et al., 1985; Yamaura et al., 1986; Nakamura et al., 1988; Ohlin et al., 1989b). Presently, this must be considered as the desired approach for the production of human monoclonal antibodies from in vitro or in vivo immunized cells (Ohlin et al., 1989a). It was worth noting that the EBV receptor (CD21) on human B cells was still expressed after a 6 day in vitro immunization, which is in contrast to previous beliefs that CD21 disappears on activated B lymphocytes. This is obviously not the case and EBV activation of in vitro immunized cells has been shown to presently be the superior method of immortalization (Ohlin et al., 1989a). Furthermore, since the treatment of PBL with leucyl-leucine methyl ester eliminates all lysosome-rich, B cell-suppressive and cytolytic cells, including natural killer cells, the outgrowth of B cell clones from this population is significantly higher, as compared to untreated cell (Table IV).

In summary, the in vitro immunized human B lymphocytes are washed and then incubated in supernatant from B95-8 cells for 2 h. The cells are washed and seeded at 5×10^4 cells/microwell, together with 10^4 irradiated PBL as feeder cells. After a culture period of normally 3–4 weeks the supernatants are tested using a solid-phase enzyme immunoassay. After expansion of cells from antibody positive wells, it is very important to test for unspecific reactivity against, e.g., plastic, bovine serum albumin, gelatin, etc. The EBV activated cells are fused as soon as possible with a

TABLE IV

B CELL TRANSFORMATION FREQUENCIES FROM EBV-INFECTED PERIPHERAL B LYMPHOCYTES, WITH OR WITHOUT LEUCYL-LEUCINE METHYL ESTER-TREATMENT, AS DETERMINED BY POISSON DISTRIBUTION CALCULATIONS

Cell pretreatment	B cells/well	Average no. of clones/well [a]
None	100	0.3
None	2500	0.8
LeuLeu-OMe	100	0.9
LeuLeu-OMe	2500	> 3 [b]

[a] Wells containing > 50 cells/well were scored as positive.
[b] Experiments giving > 95% growth positive wells.

heteromyeloma (mouse × human), which normally gives the best yield of hybridomas. The fusion ensures a more stable phenotype and an increased antibody production, as compared to the EBV lines.

A schematic representation of the approaches discussed above is shown in Fig. 2.

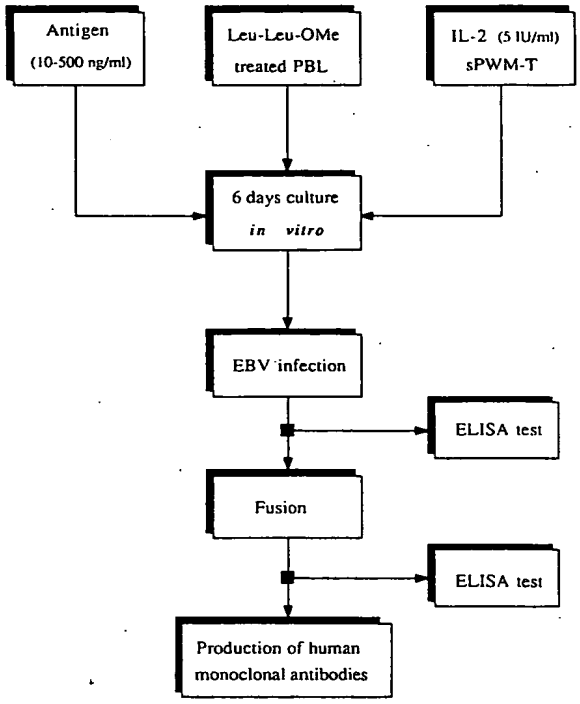

Fig. 2. A schematic presentation of the different steps involved in an optimal production technology of human monoclonal antibodies, using in vitro immunized human peripheral B cells (PBL). Leu-Leu-OMe, leucyl-leucine methyl ester; sPWM-T, supernatant from PWM stimulated (10 μg/ml for 24 h), irradiated (2000 rad) human T cells; IL-2, interleukin-2.

Stability and Ig productivity of human hybridom cell lines

The approach described above, involving in vitro immunization/stimulation and subsequent EBV activation/somatic cell fusion, has in a number of cases given a satisfactory yield of hybridomas exhibiting a long term stability in culture, i.e. > 6 months. The productivity of these cell lines has been tested and values as high as 140 μg/24 h per 10^6 cells have been recorded (Ohlin et al., unpublished data), although the productivity is normally in the range 20–50 μg/24 h per 10^6 cell. This is similar to many mouse hybridomas and hence the productivity does not seem to impose the same problem as previously, when values around 0.5–1 μg/ml were common for human hybridomas constructed using human fusion partners.

Modulation of isotype and affinity of human antibody

Isotype, affinity, and specificity of antibodies are important parameters which we need to tailor-make for individual applications of human monoclonal antibodies. Today, molecular biology has provided us with the tools necessary to perform these manipulations and there are important advantages using these approaches. First, the effector functions of the antibody molecule can be tailored as required, e.g., IgG1 and IgG3 human isotypes have been shown to be the most effective in complement and cell-mediated lysis (Bruggemann et al., 1987; Liu et al., 1987; Shaw et al., 1987) and would therefore be selected for antibody-mediated destruction of tumor cells. Second, the antibody affinity and specificity can be modulated using site-directed mutagenesis, as elegantly shown by Roberts et al. (1987) using an antibody (Gloop2) directed against a small peptide of lysozyme; this approach enables them to increase the affinity > eight times and to decrease the ability of the antibody to cross-react with closely related antigens.

Chimeric antibodies, i.e., antibodies with variable domains from mice and framework domains from humans, have recently been constructed and one example has been reported where each of the complementarity determining regions (CDR1-3) of the rodent heavy and light chains have been transplanted into a human framework; a human IgG1 antibody was modified by transplantation of only the antigen binding sites from a rat anti-human lymphocyte antibody (CAMPATH-1) (Reichmann et al., 1988). However, the grafting of rat CDRs into the human framework did not turn out to be a simple matter of replacing the six CDR1-3 of the human antibody with those from the rat and the resulting human chimeric antibody reacted poorly with the CAMPATH-1 antigen. The result suggested an error in the packing of the reshaped domains in the human FRs which resulted in an approximately 40 times lower antigen reactivity compared to the original antibody. It was only after site-directed mutagenesis that a mutant V_H was obtained which exhibited a restored antigen binding (Reichmann et al., 1988). This illustrates the important fact that hypervariable regions are not isolated within the antigen binding site but rather make a number of contacts with residues of the framework. It also points to a problem that might prevent humanizing from being the general approach to obtaining human monoclonal antibodies as once was expected (Cheetham, 1988).

However, human/human chimeric antibodies would overcome most of the problems above. If the gene segments coding for the entire V_H and V_L domains of a human monoclonal antibody, produced by in vitro immunization, were cloned into vectors containing the desired human constant gene segements, one would obtain a human/human chimeric monoclonal antibody with a specificity determined by the human immune response. We have during recent years investigated a general approach to the cloning of gene segments that code for the variable regions of human monoclonal antibodies. These gene segments could easily be amplified from single hybridoma cells, using the polymerase chain reaction and a set of degenerate primers for the 5′ end of the variable Ig region (Larrick et al., 1989). The enzymatically amplified variable gene segments could subsequently be cloned directly into a M13 phage vector or into an expression vector together with, e.g., the γ1 constant region gene segment. Presently, the production of tailor-made human/human monoclonal antibodies, constructed from PCR-amplified variable gene segments derived from in vitro immunized cells, is investigated. If routinely applicable, this approach might eventually solve most of the present problems associated with the production of human monoclonal antibodies.

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